



TITLE:

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Short title: NPH3-like proteins mediate for chloroplast movement

## **RPT2/NCH1 subfamily of NPH3-like proteins is essential for the chloroplast accumulation response in land plants**

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## Abstract

In green plants, the blue-light receptor kinase phototropin mediates various photomovements and developmental responses, such as phototropism, chloroplast photorelocation movements (accumulation and avoidance), stomatal opening, and leaf flattening, which facilitate photosynthesis. In *Arabidopsis*, two phototropins (phot1 and phot2) redundantly mediate these responses. Two phototropin-interacting proteins NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3) and ROOT PHOTOTROPISM 2 (RPT2), which belong to the NPH3/RPT2-like (NRL) family of BTB domain proteins, mediate phototropism and leaf flattening. However, the roles of NRL proteins in chloroplast photorelocation movement remain to be determined. Here, we show that another phototropin-interacting NRL protein, NRL PROTEIN FOR CHLOROPLAST MOVEMENT 1 (NCH1), and RPT2 redundantly mediate the chloroplast accumulation response but not the avoidance response. NPH3, RPT2, and NCH1 are not involved in the chloroplast avoidance response or stomatal opening. In the liverwort *Marchantia polymorpha*, the NCH1 ortholog, MpNCH1, is essential for the chloroplast accumulation response but not the avoidance response, indicating that the regulation of the phototropin-mediated chloroplast accumulation response by RPT2/NCH1 is conserved in land plants. Thus, the NRL protein combination could determine the specificity of diverse phototropin-mediated responses.



## Significance

The photoreceptor phototropin mediates various blue-light-induced responses, including phototropism, chloroplast movement, stomatal opening, and leaf flattening. Two BTB/POZ proteins, NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3) and ROOT PHOTOTROPISM 2 (RPT2), were identified as early signaling components in phototropin-mediated phototropism and leaf flattening, and a phototropin substrate, BLUE LIGHT SIGNALING1 kinase, specifically mediates the phototropin-mediated stomatal opening. However, early signaling components in the chloroplast movement remain to be determined. We found that RPT2 and the NPH3/RPT2-like (NRL) protein NRL PROTEIN FOR CHLOROPLAST MOVEMENT 1 (NCH1) redundantly mediate the chloroplast accumulation response but not the avoidance response. Our findings indicate that phototropin-mediated phototropism, leaf flattening, and the chloroplast accumulation response, but not the chloroplast avoidance response and stomatal opening, are mediated by NRL proteins.

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To adapt to a fluctuating light environment, plants evolved various light responses and photoreceptor systems. Phototropins (phot1 and phot2 in *Arabidopsis thaliana*) are blue-light receptor kinases (1) belonging to AGC kinase VIII family (named after the cAMP-dependent protein kinase A, cGMP-dependent protein kinase G and phospholipid-dependent protein kinase C) (2). phot1 and phot2 redundantly mediate hypocotyl phototropism, the chloroplast accumulation response, stomatal opening, leaf positioning, and leaf flattening, although functional differences between phot1 and phot2 exist (3-6). For example, phot2 is the main photoreceptor for the chloroplast avoidance response (7, 8) and palisade cell development (9). However, how phot1 and phot2 regulate these responses was still undetermined.

NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3) and ROOT PHOTOTROPISM 2 (RPT2) were identified through molecular genetic analyses of phototropism-deficient mutants (10, 11). NPH3 and RPT2 are founding members of the NPH3/RPT2-like (NRL) family, which consists of 30~ proteins in *Arabidopsis* (12). NRL proteins have an N-terminal BTB (broad complex, tramtrack, and bric à brac) domain and four conserved regions, I-IV (10) with region I partially overlapping the BTB domain (Fig. 1A and S1). NPH3 and RPT2 are localized on the plasma membrane, like phototropins, and interact with them (10, 13). The *nph3* mutant is completely defective in hypocotyl and root phototropism (10, 11). The *rpt2* mutant lacks root phototropism but retains partial hypocotyl phototropism under a low blue-light fluence rate (11, 13). The blue-light-induced dephosphorylation of NPH3, which is dependent on phot1, is thought to be an early event in the phototropic response (10, 14, 15). *rpt2* mutants exhibit excessive levels of blue-light-induced NPH3 dephosphorylation and have an enhanced rate of NPH3 internalization, indicating that RPT2 mediates phototropism via the regulation of the phosphorylation status and localization of NPH3 (16). Both *nph3* and

*rpt2* mutants are also impaired in leaf flattening and positioning (6, 17, 18, 19). However, *nph3* and *rpt2* exhibit normal chloroplast photorelocation movements and stomatal opening (6, 17, 20). Thus, NPH3 and RPT2 likely mediate auxin-dependent processes among phototropin-mediated responses. Indeed, phot1 and NPH3 mediate auxin transport during blue-light-induced root phototropism via the regulation of the subcellular localization of an auxin efflux transporter PIN-FORMED 2 (21). Another phototropin-interacting protein family, PHYTOCHROME KINASE SUBSTRATE, is also necessary for leaf positioning and flattening, as well as phototropism, but not for chloroplast photorelocation movements and stomatal opening (17, 22, 23). Thus, signaling pathways for chloroplast photorelocation movements and stomatal opening may be very different from those for phototropin-mediated auxin responses.

Chloroplasts move towards weak light (the accumulation response) and escape from strong light (the avoidance response). In land plants from liverwort to flowering plants, phototropin is the blue-light receptor for chloroplast photorelocation movement (24). Similar to *Arabidopsis*, the functional divergence between phototropins is found also in the fern *Adiantum capillus-veneris* and the moss *Physcomitrella patens* (25, 26). Importantly, in the liverwort *Marchantia polymorpha*, which belongs to the most basal land plant lineage, a single phototropin, Mpphot, mediates both the accumulation and avoidance responses (27). Thus, although the functional divergence of phototropins occurred during land plant evolution, phototropins intrinsically have the ability to mediate both the accumulation and avoidance responses.

How one type of photoreceptor (i.e., phototropin) mediates different physiological responses is an important theme in understanding complex light-signaling pathways in plants. Here, we report that two phototropin-interacting NRL proteins, NRL PROTEIN FOR CHLOROPLAST MOVEMENT 1 (NCH1) and RPT2, are essential for the phototropin-mediated chloroplast accumulation response. Furthermore, NPH3, RPT2,

and NCH1 are not involved in the chloroplast avoidance response or stomatal opening. Thus, the combination of NRL proteins may determine the functional specificities among phototropin-mediated responses.

## Results

**Identification of NCH1 as a phototropin signaling factor.** Because NPH3 and RPT2 are early signaling components in phototropin-mediated responses, we hypothesized that NRL proteins, other than NPH3 and RPT2, are involved in chloroplast photorelocation movements. While analyzing the T-DNA insertion mutants of some *NRL* genes, we found that the T-DNA lines of the *NRL* gene *At5g67385* were deficient in chloroplast photorelocation movements (see below in detail). Here, we named *At5g67385* as *NRL PROTEIN FOR CHLOROPLAST MOVEMENT 1* (*NCH1*), and the two T-DNA lines as *nch1-1* (SALK\_064178) and *nch1-2* (GABI\_326\_A01) (Fig. 1B). Previously, *NCH1* was identified as a plant immunity regulator gene, AtSR1 interaction protein 1 (*SRIIP1*) (28) and also named as *NPH3/RPT2-Like 31* (*NRL31*), but hereafter we call *At5g67385* as *NCH1* in this paper. *NCH1* showed a higher similarity to RPT2 than to NPH3 (Fig. S1). A phylogenetic analysis indicated that NRL proteins are classified into seven major clades and that *NCH1* belonged to the same clade as RPT2 but a distinct clade from NPH3 (Fig. 1C and S2; 12, 29). *At3g49970* (named as *NCH1-LIKE 1*, *NCL1*) was the closest paralog of *NCH1* (Fig. 1B, S1 and S2; 12, 29). However, there is no evidence of its *in vivo* expression and we did not detect any defects of *ncl1* mutant at least in chloroplast photorelocation movement and stomatal opening (see below), suggesting that the *NCL1* in *A. thaliana*, if functional, has a role other than chloroplast photorelocation movement and stomatal opening (Supplemental Text, Fig. S1 and see below).

To examine the subcellular localization of *NCH1*, we constructed transgenic *nch1-1* plants expressing the *NCH1-GFP* fusion gene under the control of the native *NCH1* promoter (*NCH1pro:NCH1-GFP*). *NCH1-GFP* fluorescence was detected in both the pavement and mesophyll cells. In both cell types, *NCH1-GFP* was localized on the plasma membrane (Fig. 2A). An immunoblot analysis using an *NCH1* antiserum

revealed smeared bands around ~60 kDa (frequently as prominent two bands) in wild-type, *phot1phot2*, and *rpt2*, but not in *nch1* mutant plants (Fig. S3A). They were detected in the microsomal fraction, similar to *phot1* (Fig. S3A and B). In an immunoprecipitation analysis of the microsomal fraction with an anti-GFP antibody, endogenous NCH1 was co-immunoprecipitated with *phot1*-GFP but not with GFP alone in a blue-light-independent manner (Fig. 2B; Fig. S3C). *In vitro* pull-down assay revealed that NCH1 directly interacted with both N- and C-terminal domains of *phot1* (Fig. 2C and D). Similar to *nph3* and *rpt2* mutant plants (10, 11), the blue-light-induced autophosphorylation of *phot1* was normal in *nch1* mutant plants (Fig. S3A and D), indicating that NCH1 is downstream of the phototropins. Consistent with the interaction between NPH3 and RPT2 (13), yeast two hybrid analysis revealed the interactions between N- and C-terminal domains of NPH3, RPT2, NCH1 (Fig. 1A), although the interaction between RPT2 and NCH1 was much weaker (Fig. S4). Collectively, NCH1 is a phototropin-interactive protein similar to NPH3 and RPT2.

**NCH1 is not involved in the hypocotyl phototropic response and leaf flattening.** We analyzed hypocotyl phototropic responses in wild-type, *nch1* (*nch1-1* and *nch1-2*), *rpt2-4* (SAIL\_140\_D03; Fig. 1A), and *rpt2nch1* (*rpt2-4nch1-1* and *rpt2-4nch1-2*). Similar to other *rpt2* mutant plants (11, 13, 20), *rpt2-4* is defective in the phototropic response, especially at higher fluence rates (Fig. S5A). The *nch1* mutant plants showed a slightly strong phototropic response compared with wild type, although the only differences between wild type and *nch1-2* in phototropic response at 4 and 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$  were statistically significant (Fig. S5A; Student's *t* test,  $0.01 < P < 0.05$  for wild type vs. *nch1-2* at 4 and 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). *rpt2nch1* mutant plants were similar to *rpt2-4* (Fig. S5A; Student's *t* test,  $P > 0.1$  for *rpt2-4* vs. *rpt2-4nch1-1* or *rpt2-4nch1-2* at all fluence rates), indicating that NCH1 is not a positive regulator of phototropin-mediated

phototropic responses.

When grown under the continuous white light, *nch1-1* mutants exhibited normal leaf flattening, like wild type (Fig. S5B). Under our light conditions, the leaves of *rpt2* mutant plants were slightly curled but leaf curling was not as severe as in the *phot1phot2* double mutants (Fig. S5B). The leaf curling of *rpt2nch1* double mutants was similar to that of *rpt2* (Fig. S5B). Thus, NCH1 is not involved in leaf flattening.

**RPT2, NCH1, and NPH3 are not involved in stomatal opening.** Recently, it was reported that *rpt2* mutant and *phot2rpt2* double mutant exhibit normal blue-light-induced stomatal opening (20), contrary to a previous study (13). We examined a possible redundancy among four *NRL* genes in stomatal opening using *rpt2nch1*, *phot2rpt2*, *phot2rpt2nch1*, and *nph3rpt2nch1ncl1* mutant plants (Fig. S6). When the blue-light-induced stomatal opening in intact leaves was measured using an infrared thermograph, a blue-light-induced decrease in leaf temperature caused by stomatal opening was found in wild type but not *phot1phot2* double mutants as described previously (Fig. S6A) (30). Consistently, blue-light-induced stomatal opening in isolated epidermal tissues was found in wild type but not in *phot1phot2* (Fig. S6B). In both assays, normal blue-light-induced stomatal opening was detected in *rpt2nch1*, *phot2rpt2*, and *phot2rpt2nch1* (Fig. S6A and B), indicating that RPT2 and NCH1 are dispensable for phototropin-mediated stomatal opening. Furthermore, *nph3rpt2nch1ncl1* quadruple mutant plants exhibited normal blue-light-induced stomatal opening (Fig. S6A and B). Blue-light-induced proton pumping (Fig. S6C) and  $H^+$ -ATPase phosphorylation (Fig. S6D), which are essential for phototropin-mediated stomatal opening (30), were also normal in the quadruple mutant plants (Fig. S6C and D). Thus, at least four *NRL* genes are not involved in phototropin-mediated stomatal opening.

**RPT2 and NCH1 are essential for the chloroplast accumulation response but not the avoidance response.** The chloroplast photorelocation movement was analyzed through the leaf transmittance of red light (31). In wild type (black in Fig. 3 and Fig. S7), weak blue light ( $3 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) induces a decrease in leaf transmittance caused by the chloroplast accumulation response whereas strong blue light (20 and  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) induces an increase in the leaf transmittance as a result of the avoidance response. After the strong blue light was turned off, a rapid decrease in leaf transmittance occurred (called the “dark recovery response”). In the *nch1* mutant plants (indigo in Fig. 3 and Fig. S7), the chloroplast accumulation response was slightly weaker compared with that of the wild type, although the difference was statistically insignificant in many experiments (Fig. 3A and B). However, the avoidance response was strongly enhanced in the *nch1* mutants (Fig. 3A and B). Not only the speed, but also the amplitude of the avoidance response was strongly enhanced in *nch1* mutants, especially at  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Fig. 3A and B; Student’s *t* test,  $P < 0.005$  for wild type vs. *nch1*). Furthermore, the dark recovery response was impaired in the *nch1* mutants (Fig. 3A and B; Student’s *t* test,  $P < 0.01$  for wild type vs. *nch1*). The *nch1incl1* mutant showed a phenotype similar to that of *nch1* single mutants (cyan in Fig. S7G and H; Student’s *t* test,  $P > 0.2$  for *nch1-1* vs. *nch1-incl1-1* at all fluence rates). However, in *rpt2nch1* (orange in Fig. 3 and Fig. S7), the increase, but not the decrease, in leaf transmittance occurred even under the weak blue-light condition (Fig. 3A and B; Fig. S7A and B), indicating that *rpt2nch1* is completely defective in the accumulation response. The enhanced avoidance response at 20 and  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  observed in *nch1* was suppressed in *rpt2nch1*. At  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ , the increase in the leaf transmittance in *rpt2nch1* was much lower than that in wild type. The avoidance response may have been saturated by the preceding light condition ( $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Contrary to a previous study that detected no defects



in chloroplast photorelocation movements in the *rpt2* mutant plants (13), we found that the *rpt2* mutant plants, *rpt2-3* (20) and *rpt2-4*, exhibited a weak accumulation response (red in Fig. 3A and B; Fig. S7A and B; Student's *t* test,  $P < 0.01$  for wild type vs. *rpt2* at  $3 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) but the avoidance response and the dark recovery response were normal (Fig. 3A and B and Fig. S7A and B; Student's *t* test, for wild type vs. *rpt2*,  $P > 0.05$  in the avoidance response and  $P > 0.2$  in the dark recovery response). Four *rpt2nch1* (*rpt2-3nch1-1*, *rpt2-3nch1-2*, *rpt2-4nch1-1*, and *rpt2-4nch1-2*) mutants displayed the same defect in the accumulation response (Fig. 3A and B; Fig. S7A and B). Furthermore, the expression of *NCH1-GFP* driven by the *NCH1* native promoter (*NCH1pro:NCH1-GFP*, abbreviated as *CCG*; cyan in Fig. S7C and D) rescued the accumulation rate in *phot2rpt2nch1* (Fig. S7C and D; Student's *t* test,  $P < 0.0005$  for *phot2-1rpt2-4nch1-1* vs. *CCG* in *phot2-1rpt2-4nch1-1* at  $3 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). *NCH1pro:NCH1-GFP* rescued *nch1* defects in the accumulation response, although the initial accumulation rate was not different among the genotypes in the dark recovery response (Fig. S7E and F; Student's *t* test,  $P < 0.05$  for *nch1-1* vs *CCG* lines). This partial rescue might be partly caused by lesser accumulation of *NCH1-GFP* in *CCG* lines (Fig. S8). Thus, *NCH1* and *RPT2* are necessary specifically for the accumulation response.

Similar to *rpt2nch1*, the *nph3rpt2nch1ncl1* quadruple mutant plants (orange in Fig. S7G and H) retained the avoidance response (Fig. S7G and H), indicating that *NPH3*, *RPT2*, *NCH1*, and *NCL1* are not involved in the avoidance response.

### ***phot2rpt2nch1* showed no chloroplast photorelocation movement like *phot1phot2*.**

To reveal the relationships of *RPT2* and *NCH1* with phototropins, the chloroplast movement in double- and triple-mutant plants between *nch1*, *rpt2*, *phot1*, and *phot2* was analyzed. *phot1rpt2* (green in Fig. S7I and J) exhibited a weak accumulation response similar to the *phot1* (cyan in Fig. S7I and J) and *rpt2* single mutants (Fig. S7I and J;

Student's *t* test,  $P > 0.3$  for *phot1-5rpt2-4* vs. *phot1-5* or *rpt2-4* at  $3 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). The *phot1nch1* (yellow green in Fig. S7I and J) displayed an enhanced avoidance response and an impaired dark recovery response similar to *nch1* but showed a weak accumulation response similar to *phot1* (Fig. S7I and J; Student's *t* test,  $P < 0.05$  for *nch1-1* vs. *phot1-5nch1-1* at  $3 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Both chloroplast accumulation and the avoidance responses in *phot1rpt2nch1* (magenta in Fig. S7I and J) were similar to those of *rpt2nch1* (Fig. S7I and J; Student's *t* test,  $P > 0.5$  for *rpt2-4nch1-1* vs. *phot1-5rpt2-4nch1-1* at all fluence rates). *phot2rpt2* (green in Fig. 3C and D) and *phot2nch1* (yellow green in Fig. 3C and D) exhibited a severe impairment of the avoidance response, similar to that of the *phot2* mutant (cyan in Fig. 3C and D), although they showed weaker accumulation responses than *phot2*, and similar to those of *rpt2* and *nch1* (Fig. 3C and D; Student's *t* test,  $P < 0.01$  for *phot2-1* vs. *phot2-1rpt2-4* or *phot2-1nch1-1* at  $3 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Prominently, the *phot2rpt2nch1* [magenta in Fig. 3C and D (and S7C and E)] completely lacked both the accumulation and avoidance responses (Fig. 3C and D), like the *phot1phot2* double mutant. These results indicated that both *phot1* and *phot2* mediated the accumulation response through RPT2 and NCH1.

**The RPT2/NCH1 homolog mediates the chloroplast accumulation response in the liverwort *M. polymorpha*.** To investigate the conserved role of NRL proteins in phototropin-mediated chloroplast movements in land plants, we searched and characterized *RPT2/NCH1* homologous genes in the liverwort *M. polymorpha*. *M. polymorpha* has seven *NRL* genes and each one *NRL* gene in seven major clades (Fig. S2). MpNCH1 belongs to a clade that includes the Arabidopsis *RPT2* and *NCH1* genes (Fig. 1B and S2), and has the same intron position as Arabidopsis *NCH1* (the third intron of MpNCH1 corresponds to the fourth intron of *NCH1*; Fig. 1A and 4A). The

amino acid sequence of MpNCH1 is similar to those of RPT2 and NCH1 (Fig. S9). We generated an MpNCH1 knockout line, *Mpnch1<sup>ko</sup>*, using the homologous-recombination-based gene-targeting method (32; Fig. 4B). In the rim region of wild-type gemmalings (the early stage of thalli developing from gemmae) that were cultured under the white light conditions for 3 days, chloroplasts covered the upper surface of the cells as a result of the chloroplast accumulation response (Fig. 4C). However, in *Mpnch1<sup>ko</sup>*, only a few chloroplasts were situated on the upper cell surface under the same conditions (Fig. 4C), and the distribution of chloroplasts was similar to that in the strong-light-irradiated wild-type cells where the chloroplast avoidance response was induced (27). When the chloroplasts that were localized on the anticlinal walls in *Mpnch1<sup>ko</sup>* were irradiated with a strong blue light using a confocal laser scanning microscope, chloroplasts escaped from the irradiated area (Supplemental movie S1), indicating that *Mpnch1<sup>ko</sup>* is deficient in the accumulation response but not in the avoidance response. The defects of *Mpnch1<sup>ko</sup>* in the chloroplast distribution were completely rescued by the expression of wild-type MpNCH1 cDNA or the NCH1-Citrine fusion gene driven under the control of the cauliflower mosaic virus 35S promoter (Fig. 4C and D). The functional NCH1-Citrine was predominantly localized on the plasma membrane (Fig. 4D). Thus, NCH1 is necessary for the phototropin-mediated accumulation response in both *Arabidopsis* and *M. polymorpha*, and phototropin-NCH1 signaling on the plasma membrane is conserved in land plants.

## Discussion

NRL proteins, such as NPH3 and NAKED PINS IN YUC MUTANTS 1 (NPY1), may mediate auxin-mediated responses via auxin transport and auxin signaling, together with AGC kinase VIIIs, such as phototropin and PINOID (33). The phototropin-NPH3 module regulates phototropic responses and leaf flattening (6, 10, 11, 17), and the

PINOID-NPY1 module regulates auxin-mediated organogenesis and root gravitropic responses (29, 34, 35, 36). Phototropin-NPH3 and PINOID-NPY1 regulate the localization of auxin efflux carrier PIN-FORMED (PIN) proteins (21, 37) and result in the activation of AUXIN RESPONSE FACTOR transcription factors (38, 39). In this study, we found that two phototropin-interacting NRL proteins, RPT2 and NCH1, mediate the chloroplast accumulation response. Because chloroplast photorelocation movement is cell autonomous and occurs even in enucleated cells (40), RPT2 and NCH1 mediate the chloroplast accumulation response independently of auxin transport and auxin-mediated transcription. Thus, the AGC kinase-NRL module can regulate other molecular mechanisms, in addition to the auxin-related mechanism, via PIN regulation.

NCH1 is specific to the chloroplast accumulation response and NPH3 is specific to phototropism and leaf flattening, but RPT2 mediates both responses. Because NCH1 and NPH3 can activate phototropin signaling pathways in the absence of RPT2 (i.e., *rpt2* mutants), RPT2 could not simply function as a molecular hub for the phototropin signaling pathways. Although RPT2 is a modulator of NPH3, which is essential for phototropism (16), the relationship between RPT2 and NCH1 in the chloroplast accumulation response is redundant. Although blue light induces the changes in NPH3 localization pattern, i.e., the internalization from the plasma membrane to cytosol (16), we could not detect any clear changes of localization pattern of NCH1 at least during 10 min of blue light irradiation in both *A. thaliana* and *M. polymorpha* (Fig. S10). Thus, blue-light-activated phototropins might mediate chloroplast accumulation response through the mechanism other than the regulation of NCH1 localization.

Previously, we identified *J-domain protein required for chloroplast accumulation response 1* (*jac1*) mutants (41) that were defective in the accumulation response and the enhanced avoidance response, like *rpt2nch1* (42), suggesting that JAC1 is a regulator

for the accumulation response, together with RPT2/NCH1. However, the phenotypes of both *jac1* and *rpt2nch1jac1* mutant plants were considerably different from that of *rpt2nch1* (Supplemental Text, S7K and L), and the amount of NCH1 was normal in *jac1* (Fig. S3D), suggesting that JAC1 has slightly different functions from RPT2 and NCH1. Nevertheless, it is important to elucidate the relationship between JAC1 and RPT2/NCH1.

An RPT2/NCH1 ortholog, MpNCH1, mediates the chloroplast accumulation response and localizes on the plasma membrane in *M. polymorpha*, indicating that the signaling mechanism for the accumulation response is conserved in land plants. Phototropins and components for the motility system of chloroplast photorelocation movements, such as CHLOROPLAST UNUSUAL POSITIONING 1 (CHUP1) and KINESIN-LIKE PROTEIN FOR ACTIN-BASED CHLOROPLAST MOVEMENT (KAC) are highly conserved from liverwort to seed plants (25-27, 43-46). However, *M. polymorpha* and other non-seed plant genome database searches identified *RPT2/NCH1*, *CHUP1* and *KAC* orthologs, but not those of *JAC1* or some other genes, in non-seed plant genome sequences. Therefore, although the photoreceptor (phototropin), phototropin-interacting protein (RPT2/NCH1), and motility system (CHUP1 and KAC) are conserved, the signal transduction pathway might have developed during land plant evolution.

In conclusion, the specificity of some phototropin-mediated signaling pathways was determined by the members and the combinations of phototropin-interacting NRL proteins. NPH3 is specific to phototropism and leaf flattening, and NCH1 is specific to chloroplast accumulation response. The involvement of NRL proteins in the chloroplast avoidance response is presently unknown. The phototropin-interacting kinase BLUE LIGHT SIGNALING1 mediates stomatal opening and is the direct substrate of phototropin (30). However, NRL proteins are not direct substrates of phototropin. In

addition to NPH3 and RPT2, we should analyze the phototropin-dependent signaling pathway taking into account NCH1.

We identified one NPH3 ortholog, MpNPH3, in the *M. polymorpha* genome (Fig. S2), implying that the divergence of NRL proteins in phototropin-dependent signaling occurred early in land plant evolution and that AGC kinase-NRL modules are an early innovation during land plant evolution.

## Materials and Methods

For the plant materials, the analysis of phototropin-mediated responses, confocal laser scanning microscopy, immunoblotting, and other methods, please see *SI Materials and Methods*.

## ACKNOWLEDGEMENTS

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## FIGURE LEGENDS

Fig. 1. Identification of NCH1 as a phototropin-signaling component. (A) Protein structure of NCH1. Four conserved regions I to IV (black boxes) and a BTB/POZ domain (black bar) are indicated. N- and C-terminal regions that are used in yeast two hybrid assay are indicated below. (B) Gene structure and mutation sites of *RPT2*, *NCH1*, and *NCLI* genes. The rectangles and the intervening bars indicate exons and introns, respectively. The white rectangles and bars in the *NCLI* gene indicate the predicted exon and intron sequences, respectively, which are similar to those of *NCH1* but were deduced to be non-coding. The positions of the T-DNA insertion sites and the point mutation are indicated. (C) A RPT2/NCH1 clade in the NRL protein phylogenetic tree. The full NRL protein phylogenetic tree is presented in Fig. S2. The number indicates the branch support value of the RPT2/NCH1 branch. Bar = 0.5 substitutions per site. AT, *Arabidopsis thaliana*; AmTr, *Amborella trichopoda*; MA, *Pinus abies*; Smoe, *Selaginella moellendorffii*; Phpat, *Physcomitrella patens*; and Mapoly, *Marchantia polymorpha*.

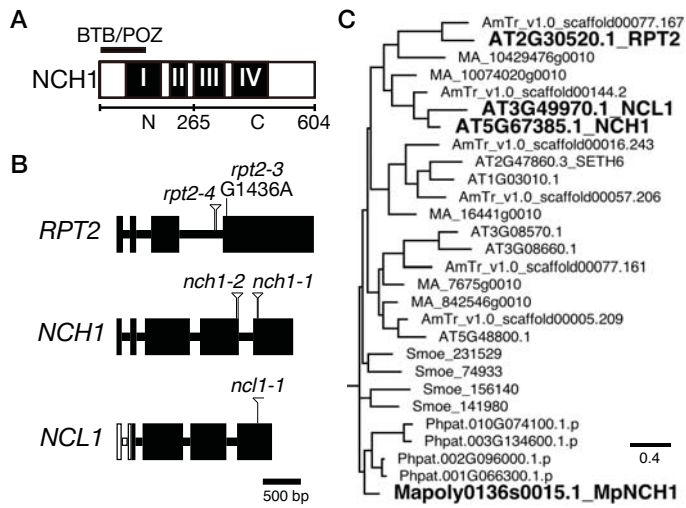
Fig. 2. (A) Plasma-membrane localization of NCH1-GFP. Images are false colored to show GFP (green) and chlorophyll (red) fluorescence. P: pavement cells, M: mesophyll cells. Bars = 10  $\mu$ m. (B) Co-immunoprecipitation of NCH1 with phot1. Microsomal fraction of the proteins from 10-day-old light-grown seedlings of transgenic GFP and phot1-GFP lines probed with anti-NCH1 or anti-phot1 antibodies. (C and D) Pull-down assay of phot1 and NCH1. FLAG- or His-tagged phot1 or NCH1 proteins were synthesized by in vitro transcription/translation reactions. The proteins bound to anti-FLAG beads were detected using anti-FLAG and anti-His.

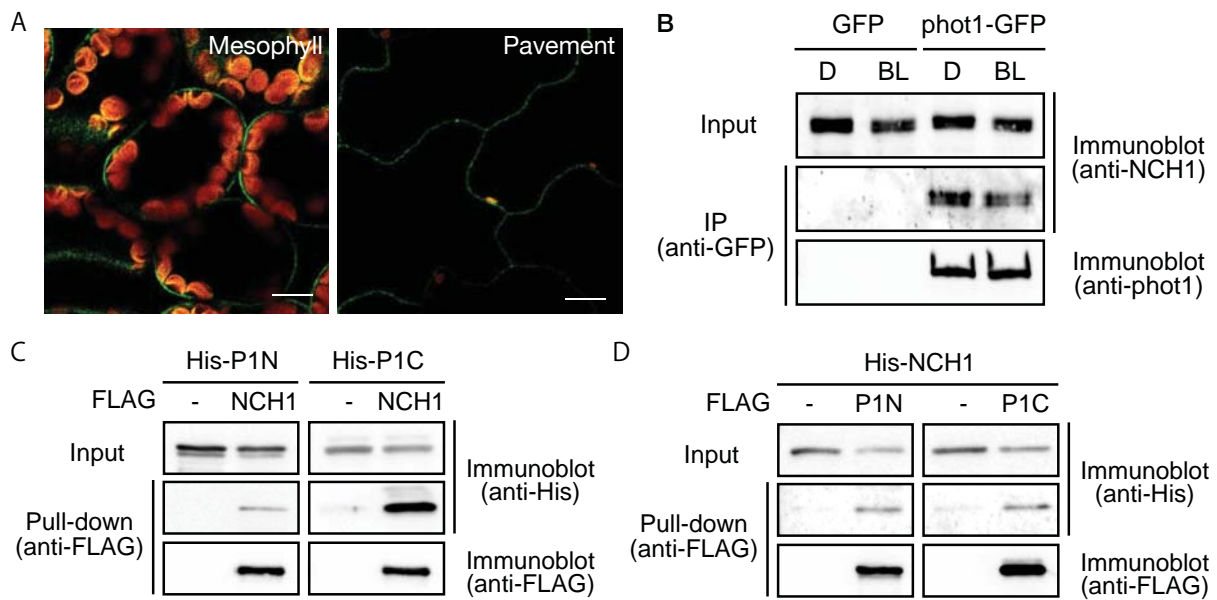
Fig. 3. Chloroplast photorelocation movements in *rpt2* and *nch1* mutant plants. (A–D)

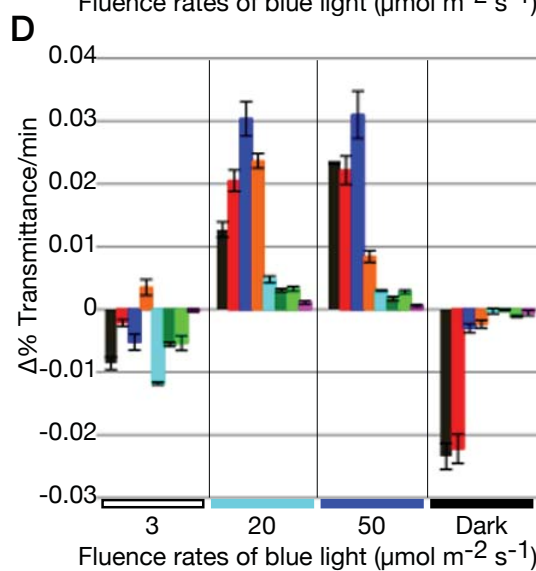
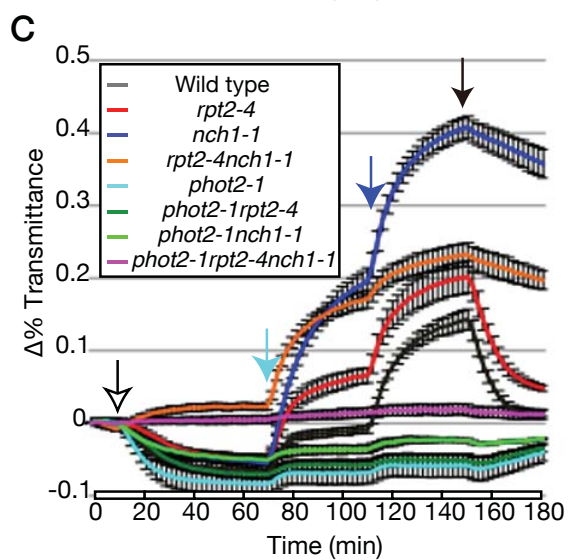
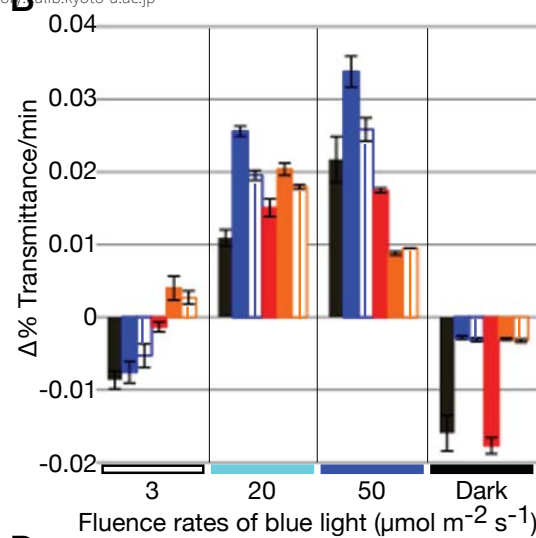
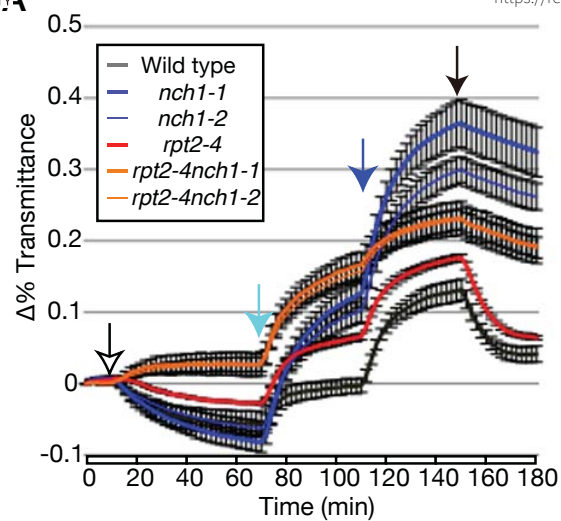
Analysis of chloroplast photorelocation movements through the measurement of leaf transmittance changes. (A and C) After a dark treatment for 10 min, the samples were sequentially irradiated with continuous blue light at 3, 20, and 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 60, 40, and 40 min, indicated by white, light blue, and blue arrows, respectively. The light was turned off after 150 min (black arrow). Data are presented as means of three independent experiments, and the error bars indicate standard errors. (B and D) Changes in leaf transmittance rates during the 2–6 min after changes in the light fluence rates (3, 20, and 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  or dark) are indicated as averages of transmittance changes for 1 min. Data are presented as means of three independent experiments and the error bars indicate standard errors.

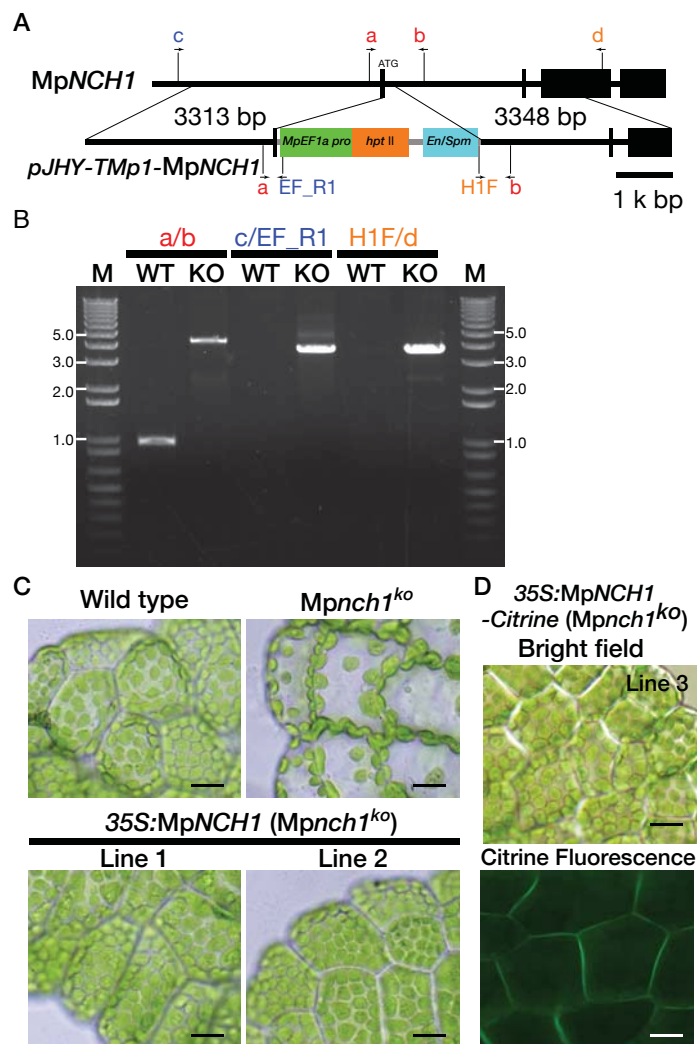
Fig. 4. MpNCH1 mediates the chloroplast accumulation response in *Marchantia polymorpha*. (A) Targeting of the MpNCH1 gene by homologous recombination. Rectangles indicate exons and the intervening bars indicate introns. A 210-bp region, including a part of the first exon, was replaced with the MpEF1a:HPTII cassette of pJHY-TMp1-MpNCH1. The positions and directions of PCR primers used in B are indicated by lines and arrows, respectively. (B) Genotyping of the Mp $nch1^{ko}$  line by PCR. The positions of the PCR primers used are shown in A. M: DNA molecular size marker, WT: wild type, KO: Mp $nch1^{ko}$ . The size of the DNA marker is indicated at both sides of the gel. (C) Chloroplast distribution under the weak continuous white-light conditions ( $\sim 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Gemmalings of wild type, Mp $nch1^{ko}$ , and two complemented Mp $nch1^{ko}$  lines were cultured under continuous white light for 3 days. (D) Plasma-membrane localization of MpNCH1-Citrine. Gemmalings of a complemented 35S:MpNCH1-Citrine Mp $nch1^{ko}$  line (Line 3) were cultured under continuous white light for 3 days. Images are false-colored to show Citrine (green). Plant samples in the bright field and epi-fluorescent images are different. Bars = 20  $\mu\text{m}$ .











# RPT2/NCH1 subfamily of NPH3-like proteins is essential for the chloroplast accumulation response in land plants

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SI Text

SI Materials and Methods

Figs. S1 to S6

Movie 1

## SI Text

### *NCLI* is likely non-functional

The chromosomal regions containing *NCHI* and *NCLI* are in an intragenome syntenic relationship (PLANT GENOME DUPLICATION DATABASE: <http://chibba.agtec.uga.edu/duplication/>). *NCHI/NCLI* gene duplication might have occurred in Brassicaceae, whereas the *RPT2/NCHI* gene duplication seems to have occurred before the separation of *Amborella* and other angiosperm (Fig. 1C). However, among the *NCLI* genes from some Brassicaceae species whose genome sequences are available, only the *Arabidopsis* *NCLI* harbors a thymine insertion in the annotated second exon (Fig. S1B). This insertion produces the premature stop codon (specific to

*Arabidopsis NCLI*) if the start codon conserved in the Brassicaceae *NCH1/NCLI* genes is used. Furthermore, there is no cDNA or EST data for *NCLI* in the TAIR database, while there are several cDNA and EST clones for *NCH1*. Additionally, an *nchl* T-DNA line (Fig. 1B) showed the wild-type phenotype (cyan in Fig. S7G and H; Student's *t* test,  $P > 0.1$  for wild type vs. *nchl-1* at all fluence rates) and the *nchl1* double mutant resembled the *nchl* single mutant (cyan in Fig. S7G and H; Student's *t* test,  $P > 0.2$  for *nchl-1* vs. *nchl-1nchl-1* at all fluence rates). Furthermore, *nph3rpt2nchl1nchl1* quadruple mutant plants exhibited normal blue-light-induced stomatal opening (Fig. S6). Thus, *NCLI* might regulate other responses if functional.

### The phenotypes of *rpt2nchl1* and *jac1* mutants are similar but distinct

The *J*-domain protein required for chloroplast accumulation response 1 (*jac1*) mutants (41) share common phenotypes, including the defective accumulation response, the enhanced avoidance response (the magnitude), and the impaired dark recovery response (42) with *rpt2nchl1*, suggesting that RPT2 and NCH1 mediate the accumulation response through the regulation of JAC1. However, a pair-wise mutant analysis indicated that the *jac1* phenotypes (cyan in Fig. S7K and L) were somewhat different from those of *rpt2nchl1*. Although the magnitude of the avoidance response increased in *jac1* and *rpt2nchl1*, the avoidance response was much stronger at  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$  in *rpt2nchl1* compared with *jac1* (Fig. S7K and L; Student's *t* test,  $P < 0.01$  for *rpt2-4nchl1-1* vs. *jac1-1* at  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). At 20 and  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ , the avoidance response was saturated in *rpt2nchl1* but not in *jac1* (Fig. S7K and L). Furthermore, *rpt2nchl1jac1* (red in Fig. S7K and L) exhibited a weaker avoidance response than *rpt2nchl1* (Fig. S7K and L; Student's *t* test,  $P < 0.001$  for *rpt2-4nchl1-1* vs. *rpt2-4nchl1-1jac1-1* at  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), consistent with *jac1* being defective in the regulation of actin filaments under the high-light condition (47). We have never detected

the interaction between NCH1/RPT2 and JAC1 in yeast two hybrid assay. Our findings suggest that JAC1 has some functions that are independent of RPT2 and NCH1.

## SI Materials and Methods

### Arabidopsis lines and the growth condition

The wild-type and mutant lines have a Columbia *gll* background. Arabidopsis cultures were performed as described previously (41). Briefly, seeds were sown on 0.8% agar medium containing 1/3 strength Murashige & Skoog inorganic salt and 1% sucrose, and cultured under white light at ca.  $\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$  (16h) / dark (8h) cycle at 23°C in an incubator. SALK and SAIL T-DNA knockout lines, SALK\_064178 (*nch1-1*), SALK\_021412 (*ncl1-1*), and SAIL\_140\_D03 (*rpt2-4*), were provided by ABRC. *nch1-2* is a GABI-Kat line, 326\_A01 (48). *phot1-5* (49), *phot2-1* (7), *nph3-6* (10), *rpt2-3* (20), and *jac1-1* (41) were described previously. Double, triple, and quadruple mutants were generated by genetic crossing. Transgenic GFP and phot1-GFP lines were described previously (30).

### Phylogenetic analyses of RPT2 and NCH1

Amino acid sequences of the full length NRL proteins were aligned using the MUSCLE program (50) implemented in Geneious software (version 6.1.8; Biomatters; <http://www.geneious.com/>) with default parameters. Amino acid sequences for the conserved BTB/POZ domain and DVI regions were extracted and combined. Unaligned gaps were removed from the resulting alignment using Gblocks ([http://molevol.cmima.csic.es/castresana/Gblocks\\_server.html](http://molevol.cmima.csic.es/castresana/Gblocks_server.html)). The Bayesian phylogenetic tree construction was performed using MrBayes 2.0.9 (51).

### Generation of transgenic *Arabidopsis* plants

To construct a vector for *NCH1pro:NCH1-GFP*, the *NCH1* gene fragment including a

2,568-bp 5' sequence (before the start codon) and the gene body region that contained the open reading frame but lacked the stop codon, was cloned into the *Pst*I and *Kpn*I restriction enzyme sites of the *pPZP221/35S:GFP-nosT* binary vector (52). The latter was constructed by cloning the *GFP* cDNA into the *pPZP221/35S-nosT* binary vector (53). The *nchl-1* mutants were transformed with *pPZP221/NCH1pro:NCH1-GFP-nosT* by the floral-dipping method using *Agrobacterium tumefaciens* (GV3101::pMP90).

### Confocal laser scanning microscopy

To observe the subcellular localization of NCH1-GFP in *A. thaliana* and MpNCH1-Citrine in *M. polymorpha*, a confocal microscope (C2+; Nikon, Tokyo, Japan) was used. Fluorescence was observed at 499–529 nm for fluorescent proteins and 553–618 nm for chlorophyll autofluorescence.

### Immunoblot analysis

An NCH1 rabbit polyclonal antibody was raised against a purified His-tag-NCH1 C-terminal domain (328–604) fusion protein (eurofins genomics, Tokyo, Japan). The phot1 antibody was described previously (46). Seedlings were homogenized in extraction buffer [50 mM Mops-KOH (pH 7.5), 100 mM NaCl, 2.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 10  $\mu$ M leupeptin, and phosphatase inhibitor cocktail (Nacalai tesque, Kyoto, Japan)]. After centrifuging three times at  $12,000 \times g$ , the supernatants (total protein fraction) were quantified using the Bio-Rad Protein Assay (Bio-Rad, CA, USA). Total protein homogenates were ultracentrifuged at  $100,000 \times g$  for 1 h at 4°C. The resultant supernatants and pellets were used as soluble and microsomal protein fractions, respectively. An immunoprecipitation analysis with anti-GFP was performed as previously described (30).



### ***In vitro* pull-down assay**

*In vitro* pull-down assay was performed as previously described (30). FLAG- and His-tagged protein pair were synthesized using an *in vitro* transcription/translation system (BioSieg, Tokushima, Japan) and kept on ice for 10 min in a binding buffer containing 20 mM Tris-HCl (pH 7.4), 140 mM NaCl and 0.1% Triton X-100. After brief centrifugation, the supernatants were incubated with anti-FLAG Ms agarose (Sigma-Aldrich, MO, USA) for 1 h. The bound proteins were used for immunoblotting analysis using anti-FLAG (Sigma-Aldrich) and anti-His antibodies (GeneScript, NJ, USA).

### **Yeast two hybrid assay**

The binding domain (bait) or the activation domain (prey) plasmids containing the genes for NCH1, RPT2, and NPH3 were constructed by subcloning the cDNAs into the pGBKT7 or pGADT7 vectors, respectively (Matchmaker<sup>TM</sup> Yeast Two-Hybrid System; Takara Bio Inc., Kusatsu, Japan). The bait and prey plasmids were transformed into the yeast strain AH109 and Y187, respectively. The mated transformants were selected on SD agar media without Leu and Trp and spotted on to the indicated media.

### **Analysis of hypocotyl phototropic responses**

The phototropic responses were analyzed as previously described with some modifications (54). The sterilized seeds were sown on 1/3-strength Murashige & Skoog agar plates without sucrose. After the cold treatment at 4°C in the darkness for 2 days, the seeds were irradiated with white light ( $\sim 80 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 3 h and then grown vertically in the dark for 54 h at 23°C. The etiolated seedlings were irradiated for 12 h with unilateral blue light (at 0.04, 0.4, 4, or  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) using blue light-emitting diodes. The hypocotyl curvature was measured using the Image J program (National

Institutes of Health, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>).

### Measurement of stomatal responses to blue light

Thermal imaging was carried out using TVS-8500 (NEC Avio Infrared Technologies, Tokyo, Japan) (30). Subtraction images were prepared using PE Professional software (NEC Avio Infrared Technologies, Tokyo, Japan). The stomatal aperture in the abaxial epidermis was measured as described previously (5). Briefly, epidermal strips were incubated in 5 mM MES-Bis-Tris propane (pH 6.5), 50 mM KCl and 0.1 mM  $\text{CaCl}_2$  under red light ( $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) superimposed by blue light ( $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 2 h at 24°C. Blue light-dependent  $\text{H}^+$  pumping from guard cell protoplasts was determined with a glass pH electrode (5). The reaction mixture (0.8 mL) contains 0.125 mM MES-NaOH (pH 6.0), 1 mM  $\text{CaCl}_2$ , 0.4 M mannitol, 10 mM KCl, and guard cell protoplasts (50  $\mu\text{g}$  proteins). Guard cell protoplasts were prepared enzymatically from leaves (55, 56). The phosphorylation of a penultimate Thr residue of the  $\text{H}^+$ -ATPase was determined by protein blotting using GST-14-3-3 (57). An immunoblot analysis was carried out using anti- $\text{H}^+$ -ATPase antibodies (57).

### Analyses of chloroplast photorelocation movements

To detect chloroplast photorelocation movements in *A. thaliana*, the measurement of leaf transmittance changes was performed as described previously (31). Detached third leaves from 16-day-old seedlings were placed on 1% (w/v) gellan gum in a 96-well plate. Leaves were dark-adapted at least for 1 h before transmittance measurement. In *M. polymorpha*, chloroplast distribution and Citrine fluorescence were observed using a fluorescent microscopy (Axiophot; ZEISS, Germany). For the observation of the chloroplast avoidance response in *Mpnchl<sup>ko</sup>*, a confocal microscope (SP5; Leica Microsystems, Wetzlar, Germany) was used as described previously (58).

### Growth conditions of *M. polymorpha*

The wild-type male accession of *M. polymorpha*, Takaragaike (Tak)-1, was used. Plants were cultured on one-half-strength Gamborg's B5 agar medium at 22°C under continuous white light ( $\sim 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ).

### Targeted gene knockout and genetic complementation of *MpNCHI*

The 5' and 3' homology arms [−3,313~+57 (3370 bp) and +268~+3,615 (3348 bp) from the start codon, respectively] of the *MpNCHI* gene were cloned into the *PacI* and *AscI* sites of pJHY-TMp1 (32), respectively. Tak-1 × Tak-2 F<sub>1</sub> spores were used for *Agrobacterium*-mediated transformation as described previously (32). The selection and PCR screening of *MpNCHI* knockout plants were performed as described previously (32). Briefly, hygromycin-resistant plants were screened by PCR with the primer pair 5'-AAGGAGGAGGAACGAACCAT-3' (a) and 5'-CGAATCTATCCCCACGAAT-3' (b). Putative *MpnchI*<sup>ko</sup> lines were confirmed using PCR with primers outside of the 5'- or 3'-homologous arms (c, 5'-CCCACTCACCTTTTCCACAT-3' or d, 5'-GTGTTCTTCTCAGGCGGAAG-3', respectively) and inside the *MpEFpro::hptII* cassette (EF\_R1, 5'-GAAGGCTTCTGATTGAAGTTTCCTTTTCTG-3' for the 5'-arm and H1F, 5'-GTATAATGTATGCTATACGAAGTTATGTTT-3' for the 3'-arm).

To generate the complementation construct, *MpNCHI* cDNA PCR fragments with or without a stop codon were cloned into pENTR/D-TOPO (Life Technologies, MA, USA). The resulting *MpNCHI* cassette was subcloned into a binary vector pMpGWB302 or pMpGWB306 (59) using LR clonase II (Life Technologies, MA, USA), resulting in the cauliflower mosaic virus *pro35S:MpNCHI* or *pro35S:MpNCHI-Citrine* binary vector, respectively. These binary vectors were introduced into regenerating thalli of *MpnchI*<sup>ko</sup> as described previously (60).

## Supplementary Figure Legends

Fig. S1. NRL protein sequence alignment. (A) The protein sequence alignment was performed using the MUSCLE program (<http://www.ebi.ac.uk/Tools/msa/muscle/>). Regions colored red, blue, orange, and green indicate the four conserved sequence domains (I–IV, respectively) defined by Motchoulski and Liscum (10). Upper red lines indicate the position of the BTB/POZ domain. A lower black line indicates the deletion in the conserved N-terminal region specific to Arabidopsis NCL1. NPH3, At5g64330; RPT2, At2g30520; NCH1, At5g67385; and NCL1, At3g49970. (B) Alignment of Arabidopsis *NCH1* and *NCL1* gene sequences. The start codons are colored orange. The 5' region of the start codon of the Arabidopsis *NCL1* gene is highly similar to *NCH1* and Brassicaceae *NCL1* exon1 and exon2 sequences but a thymine insertion (red) produces a premature stop codon (red bold). *AlNCL1*, *Arabidopsis lyrata NCL1*, XM\_002866659; *EsNCL1*, *Eutrema salsugineum NCL1*, XM\_006404018; *CrNCL1*, *Capsella rubella NCL1*, XM\_006290276; and *BrNCL1*, *Brassica rapa NCL1*, XM\_009117436.

Fig. S2. The Bayesian phylogenetic tree of NRL proteins. The parameters used were as follows: preset amino acid model program, mixed (LG, blosum, and wag); length of set rates, inverse-gamma; number of gamma categories, 4; Markov chain Monte Carlo start tree, random; number of generation, 1,000,000; number of runs, 2; number of chains, 4; heated chain temperature, 0.2; sample frequency, 200; and burn-in fraction, 0.25. klf100053\_0080 were used as the outgroup. The numbers at the nodes indicate posterior probabilities. Bars indicate substitution values per site. Both RPT2/NCH1 and NPH3 clades are indicated (red box). Seven *Marchantia* and four Arabidopsis NRL proteins (NCH1, RPT2, NCL1, and NPH3) are bold-faced. AT, *Arabidopsis thaliana*; AmTr, *Amborella trichopoda*; MA, *Pinus abies*; Smoe, *Selaginella moellendorffii*; Phpat,

*Physcomitrella patens*; Mapoly, *Marchantia polymorpha*; and kfl, *Klebsormidium flaccidum*.

Fig. S3. An immunoblot analysis of NCH1 proteins. (A) Immunoblot analysis of phot1 and NCH1 proteins. Microsomal fraction of the proteins (corresponding to 50  $\mu$ g total proteins) were used for an immunoblotting analysis with phot1 and NCH1 antibodies. The 13-day-old seedlings were dark adapted for 24 h (D) and then irradiated with 6.4  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> of blue light for 1 h (BL). An asterisk indicates the position of a non-specific protein band. (B) Total protein extracts (T) were fractionated into soluble (S) and microsomal (M) fractions by ultracentrifugation (100,000  $\times$  g, 1 h, 4°C). Immunoblotting was performed using phot1 and anti-NCH1 antibodies. (C) Microsomal fraction of the proteins from 10-day-old light-grown seedlings of transgenic GFP, phot1-GFP, and *nch1-1* mutant plants probed with anti-NCH1 or anti- H<sup>+</sup>-ATPase antibodies. H<sup>+</sup>-ATPase is a marker for microsomal proteins. (D) Immunoblot analysis of phot1 and NCH1 proteins in *jac1* mutant plants. Details are same as A.

Fig. S4. Yeast two hybrid analysis. Yeast cells expressing the indicated bait and prey vectors were cultured on SD agar media supplemented with a mixture of the appropriate amino acids without Leu and Trp (-LT), His, Leu, and Trp (-HLT), or Ala, His, Leu, and Trp (-AHLT). Yeast cells were spotted with serial dilution (1, 1/10, and 1/100 from left). Yeast cells expressing BD-RPT2 C/AD-NPH3 N, BD-RPT2 C/AD-RPT2 N, or BD-NCH1 C/AD-NPH3 N could grow well on both -HLT and -AHLT media, indicating that RPT2 C-NPH3 N, RPT2 C-RPT2 N, or NCH1 C-NPH3 N interactions is strong. Conversely, yeast cells expressing BD-NCH1 C/AD-NCH1 N could grow well on both -HLT but very weakly on -AHLT media. Yeast cells expressing BD-RPT2 C/AD-NCH1 N could grow weakly on -HLT media. Thus, RPT2 C-NCH1 N or NCH1

C-NCH1 N interaction is weak. Yeast cells containing empty BD or AD vectors could not grow on both –HLT and –AHLT media, indicating that the interactions detected here are specific.

Fig. S5. Hypocotyl phototropic response and leaf flattening in *rpt2* and *nch1* mutant backgrounds. (A) The phototropic response of the hypocotyls in etiolated seedlings. The hypocotyl curvature was induced by unilateral irradiation with blue light at the indicated fluence rates in the 3-day-old etiolated seedlings for 12 h. Data are presented as means of three independent experiments, and the error bars indicate standard errors. (B) Leaf-flattening response. After seeds were sown on vermiculite soil, plants were grown under continuous white-light conditions ( $\sim 70 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 25 days at 22°C. Bar = 1 cm.

Fig. S6. *nph3rpt2nch1ncl1* quadruple mutants are not impaired in the blue-light-induced stomatal opening.

(A) Thermal image of a blue-light-dependent leaf temperature decrease. Arabidopsis wild-type and mutant (right panel) plants were illuminated with red light (RL:  $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 50 min and then a weak blue light (BL:  $5 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was superimposed. Subtractive images (left panel) were obtained by subtracting an initial thermal image (prior to blue light exposure) from an image taken 15 min after blue light exposure. The bar represents 1 cm. (B) Light-dependent stomatal opening in the epidermis. Epidermal strips from dark-adapted plants were illuminated with red light (RL:  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and blue light (BL:  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 2 h. Bars represent means  $\pm$  standard deviations ( $n = 75$ , pooled from triplicate experiments). (C) Blue-light-dependent  $\text{H}^+$  pumping in guard cell protoplasts from wild-type and *nph3rpt2nch1ncl1* quadruple mutant plants. Guard cell protoplasts were illuminated by red light ( $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 2 h, and a

pulse of blue light ( $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ , 30 s) was applied as indicated. (D) Blue light-dependent phosphorylation of the  $\text{H}^+$ -ATPase in guard cell protoplasts. The phosphorylation of the  $\text{H}^+$ -ATPase was determined by protein blotting using a GST-14-3-3 protein. The total  $\text{H}^+$ -ATPase proteins were detected by immunoblotting using an anti- $\text{H}^+$ -ATPase antibody.

Fig. S7 Chloroplast photorelocation movements in *rpt2* and *nchl* mutant backgrounds. (A–L) Analysis of chloroplast photorelocation movements through the measurement of leaf transmittance changes in the indicated lines. (A, C, E, G, I, and K) After dark treatment for 10 min, the samples were sequentially irradiated with continuous blue light at 3, 20, and  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  for 60, 40, and 40 min, as indicated by white, light blue, and blue arrows, respectively. The light was turned off at 150 min (black arrow). Data are presented as means of three independent experiments, and the error bars indicate standard errors. (B, D, F, H, J, and L) Changes in leaf transmittance rates over 2–6 min after changes in the light fluence rate ( $3, 20, \text{and } 50 \mu\text{mol m}^{-2} \text{s}^{-1}$  or dark) are indicated as percentages of transmittance changes for 1 min. Data are presented as means of three independent experiments, and the error bars indicate standard errors. (A and B) Analysis in two *rpt2* alleles. (C and D) The *NCH1pro:NCH1-GFP* transgene (*CCG1-2*) rescued the impaired chloroplast accumulation response in *phot2-1rpt2-3nchl-1*. (E and F) Partial rescue of the *nchl* mutant phenotype in *NCH1pro:NCH1-GFP* transgenic lines (*CCG1-2*, *CCG2-8*, and *CCG5-5*). (G and H) No detectable involvement of four NRL proteins in the chloroplast avoidance response. (I and J) Genetic interactions between *PHOT1* and *RPT2/NCH1*. (K and L) Comparison of phenotypes between *rpt2nchl* and *jac1* mutants. Data are presented as means of three independent experiments (five experiments in A and B, and four experiments in I and J) and the error bars indicate standard errors.

Fig. S8. An immunoblot analysis of NCH1-GFP proteins. The 50  $\mu$ g total proteins extracted from 15-day-old seedlings were used for the immunoblotting analyses with NCH1 and phot1 antibodies. A star indicates full-length NCH1 in wild type. Black and white circles indicate full-length NCH1-GFP and the degradation products, respectively.

Fig. S9. The sequence alignment of *M. polymorpha* and Arabidopsis RPT2/NCH1 subfamily proteins. The protein sequence alignment was performed using the MUSCLE program (<http://www.ebi.ac.uk/Tools/msa/muscle/>). Regions colored red, blue, orange, and green indicate the four conserved sequence domains (I to IV, respectively) defined by Motchoulski and Liscum (10). Upper red lines indicate the positions of the BTB/POZ domains. A bottom black line indicates the deletion in the conserved N-terminal region specific to Arabidopsis NCL1. RPT2, At2g30520; NCH1, At5g67385; and NCL1, At3g49970.

Fig. S10. No apparent light-induced changes in NCH1 localization pattern. A part (indicated by white rectangles) of pavement (A) and mesophyll cells (B) of *A. thaliana* *NCH1pro:NCH1-GFP* line or cells of *M. polymorpha* 35S:MpNCH1-Citrine line (C) were irradiated with blue laser (488 nm, ca. 1000  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) for 10 min. ch: chloroplasts. Bars = 10  $\mu$ m.

### Supplementary Movie Legends

Movie 1. This time-lapse movie shows the chloroplast avoidance response induced by a blue laser in the *M. polymorpha* Mpnch1<sup>ko</sup> line. A piece of weak light-adapted *nchl*<sup>ko</sup> gemmaling was placed into a cuvette that was composed of two round cover slips that were supported by a ring-shaped silicon-rubber spacer. The time-lapse images



(maximized with three images in a 1.2- $\mu\text{m}$  depth) were collected at approximately ~01:06 (mm:ss) intervals and played back at five frames per second (fps). The total elapsed time is 33:17 (mm:ss). The images are false-colored to indicate chlorophyll (red) fluorescence and bright transmittance field (gray). The region indicated by a blue rectangle (15  $\mu\text{m}$   $\times$  50  $\mu\text{m}$ ) was irradiated using 458-nm laser scans during the intervals between the image acquisitions to induce the avoidance response. Scale bar = 25  $\mu\text{m}$ .

## A

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NPH3 1 MMWESESDGGVCGGGGREYGDGGLSSNKGGVKTDGFELRGQSWFVAIDIPSDLLVKIGDMNHLHKVPLSSSEKNRLIVE-SKDPPTTIIIDDL
RPT2 1 MATECKNPINMNSSSSLA-----RTGQWFFSODIPDVIVVGEANFSLHKFPLVARSNIKLLIME-SKDSVIRINISDI
NCH1 1 -----MSAKKDDLSSAM-----RTSEWSSCEVSSDVIVVGEASFSLHKFPLSKCGFIKKLSESSKDSSTWIKLIPDI
NCL1 1 -----MUEKLSFLLHKFPLVSKCGFIKKLSESSNDSN--IIRIPDF

NPH3 100 PGGPEAFELAAKFCYGVVVDLTATNLSGLCAAEYLEMTEHLEECNLIFTEAHLSYVLLSSWRDSTIVLKSCEKLSFMAENIQVRRCSSESIANKAGSN
RPT2 78 PGGPEAFELAAKFCYGVAFITIVONIAALCAAELEMTIKYCDNNLAGRTQDPLSQVALSSLSGAILVLKSCGELLPESRDGVRRCVIVVGAIAK--
NCH1 74 PGGPEAFELAAKFCYGVNFMSTENIAMLRCAAEYLEMTEHHSVENLVRAEAMLEVALKSLSSSTIVLHKSEKLLPIAERVLKLSRCIDATAYMT--
NCL1 41 PGGPEAFELVTKFCYDLSFETINTENIAMLRCAAEYLEMTEHHSVENLVETIEVLLNEVLLKSLSKSVLVLOKSQDLLPIAERVLVRCIDSIAYAT--

NPH3 200 PKGIRWAYTGKAPSPSTTNFAGSSPRWNESKDSFFLCSPS-----RNTNSQPVPPDWWFEDVSLIRIDHEVRVITAKVKGMLFELLGAVIMHY
RPT2 176 -----NEAME-----PCRTG-PNWWTEELCLLDVDFESDVSSMKQRGVRRPSSLASATITY
NCH1 172 -----QESHFCCSPSSSNSNGNEVVVQCSKQPVVDWWAEDLTVLIRIDSEGRVLIAMARGFKOGLGPVIMLY
NCL1 139 -----QES-----QSNEDVDWWAEDLAVLRIDMEREVLIAMARGFKRYSGLGPVIMLY

NPH3 289 AKKMLRGLRLEGGVLAIPAMSSATCGGLGGDEMSISCGSNSGGSSGPDWKGGGLHMLVLSAGKTNCHQSVACLAGLGISPKQORMIVESLISITFPCK
RPT2 226 TRKSLRLVLR-----HSGEGKMY-----SDPGDNSSDERS-----QORDVQSVISVLLPSK
NCH1 240 AKKSLRGL-----HFGKGMK-----RTPKQEH-----EKRIILEIVSLLPREK
NCL1 188 AKKMLRGLVR--F--NFLTEQCDIFGKAK-----KNEBOBH-----EKRIILEIVSLLPREK

NPH3 389 DSVLCPSFLRLRLRAANMKVAPALITLEKRVNCGEQAHTDOLLIPSTNNKGB-TMYDVLVORLLEHIVQBOTEGSSPSRMSPPSPQSAMYADIP--GN
RPT2 274 GLFVNVFCSLLRCAMFLDTSTCTCNLEKRSIVLEHVSDDLLIPSTYQGE-RLLDLEVRRTISARVKBKNYGV-----F--
NCH1 281 NANSVSFLSMLLRAAIFLETTVACRLOLENRMGLQGLQAVLDDLLIPSYSTFGDHSNFTVQRIILMNNLEFE-VEGV-----F--
NCL1 240 NSVSVSFLSLLRAAIFLETTVACRLOLEKRMGLQGLQAVLDDLLIPSYSTFGDNTMLDQDTVQRIILMNNLEFE-VEG-----F--

NPH3 488 NNNGGGCGNNQNAKMRVAVLVDSYILEVADORNLELKFQVLBAALPESARTCDGYYRAIDSYLKAHPLSEHERKRICRMDCQKLSMDACMHAAQN
RPT2 353 NCGDNNNGEVCVSIVORVAKIVDSYLAETIAYGLTISKFNALANLVKSARKSDPLYYRAIDILKAHPNILEEREKVCSSMDPIKLSYDARIHAAQN
NCH1 362 NNGVDLAS-----DNERVGLLENVYAEIASDRNVSLKFIGLAELIPEQSRVTEGMYRAVDYLKAHPNNSVERKKVCSLMDCQKLSREACAHAAQN
NCL1 317 NNSVDLAS-----DGLMELMITYLAEIASDRNINFAKFIGEACIPEKQSR-----MYRAIDILKHPNISEVERKKVCSLMDCQKLSRDYVAAHAAQN

NPH3 588 RRLFRVIVVQVLSQVKISNAIVANTLKESTTLGEAMGTYQEMINRKTTEATPQSQ--EGWAAAKKLIITLKGELTETKIVYELONEMVVMQGE
RPT2 452 RRLFRVIVHALYVQIKLSGVAFEEHRAV-----VLFEAQKRSQLOADITAKENEAESPLKAKKYVSDNQNI-----KIG
NCH1 457 DRLPVETIVQVLYEQQLRGEVTFNDSSPA-----SPFEPAAVLPFKLSSY--DELKLRKENCQKLELLKMKMKKEPEKES--KATS
NCL1 403 DRFQ-----ENLSNDSSPA-----PATAEN--LSPPELSSYK--NELSKIINENCYKLELLKMKMKKEPEKES--RAF

NPH3 686 RRGKVKVNISSSAMNSGWRKLSKLLKSGQESHDSGGGEQAC-----VD---PPPKRPRRNRNSIS
RPT2 529 AGAESNSNSIVSS-----KSKKHIFFSV-SKKLGKL-NPKKNGSKDTSHEIDLGVDITKPRRRRFSIS
NCH1 542 SSTISNPSSESTASTGKFEPRKSPINSV-SKKLGKL-NPFS-----ITPYNCGRIKEPRDRRHSIS
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## B

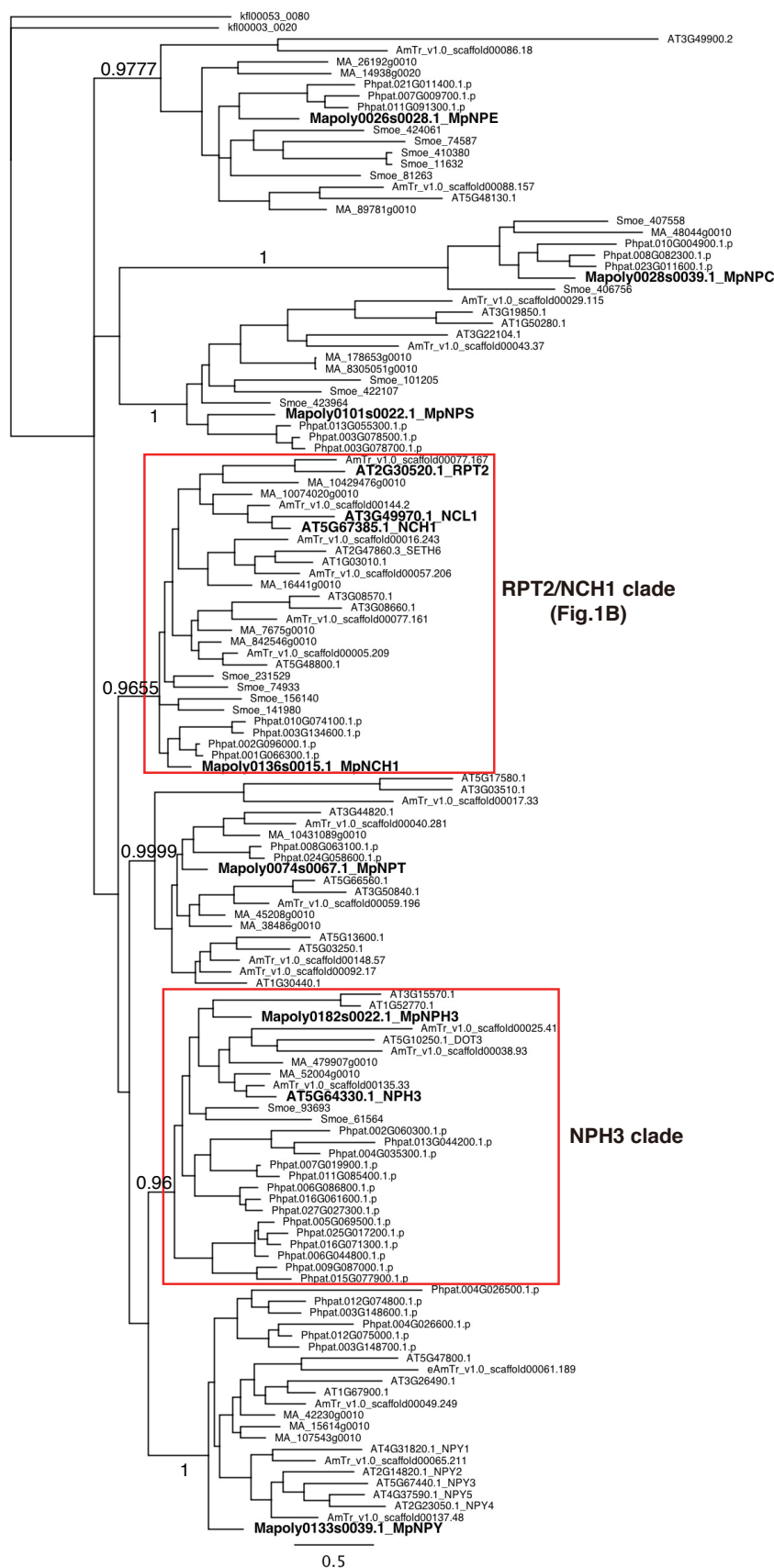
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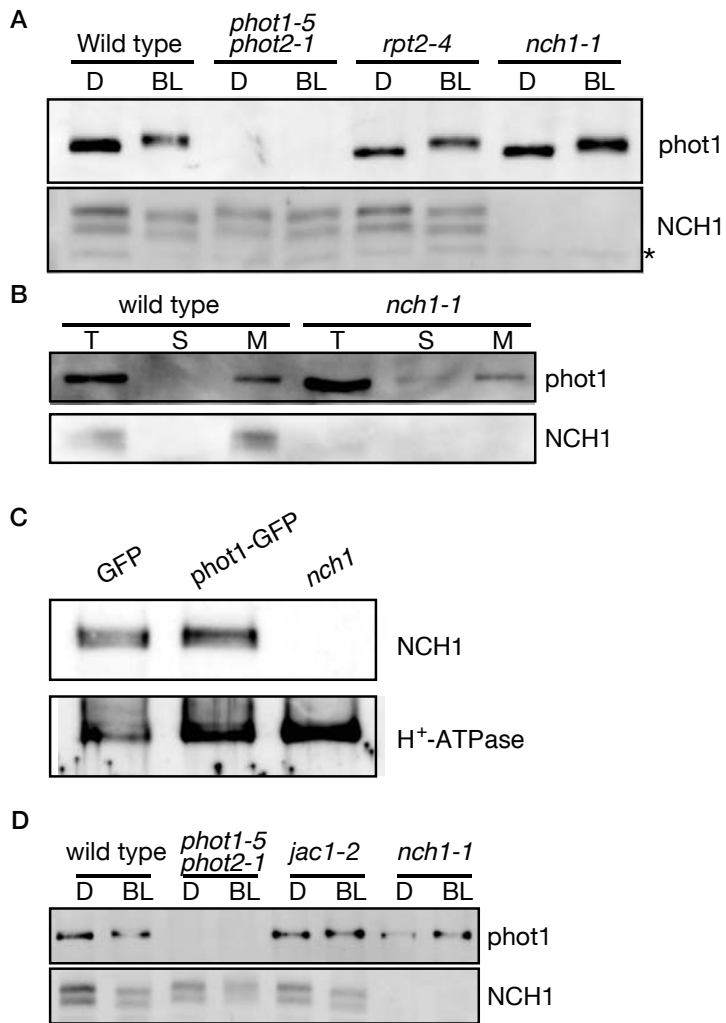
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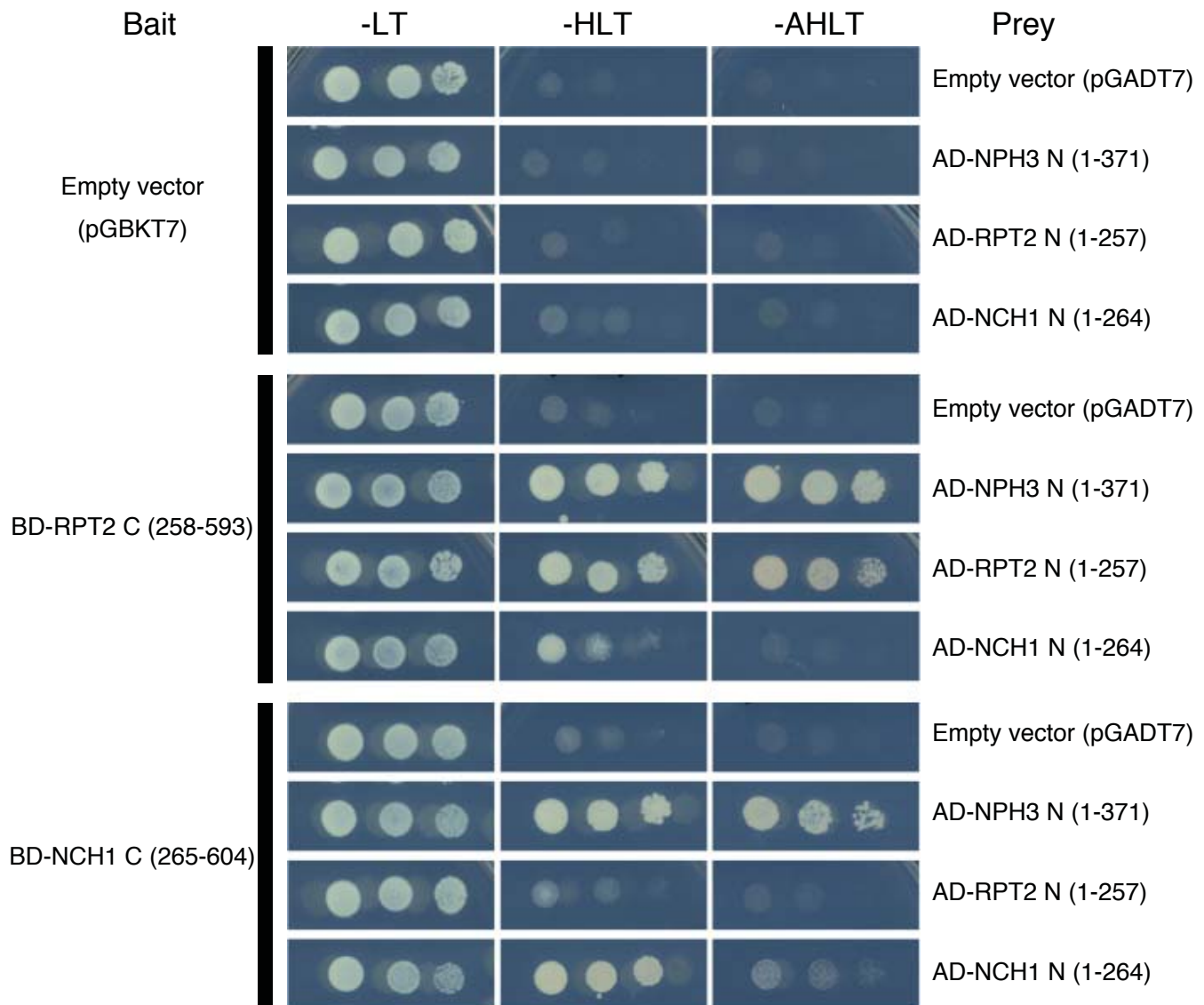
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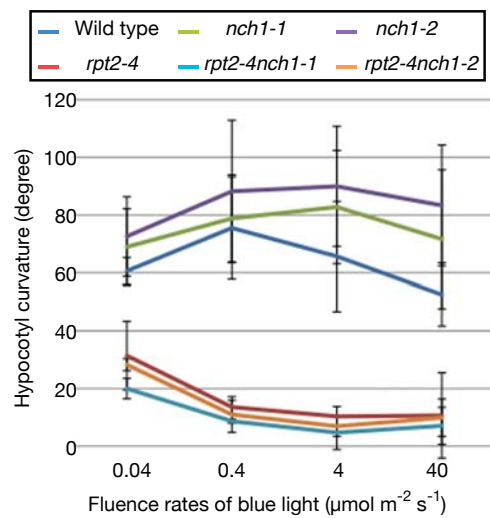
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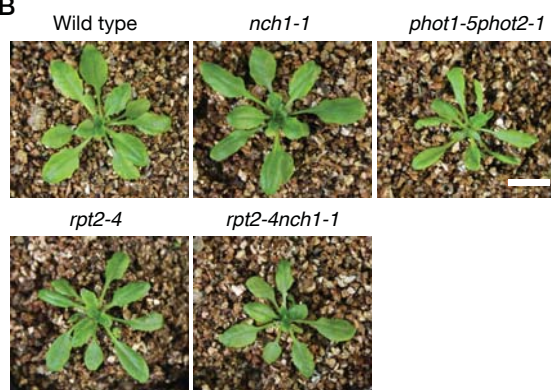


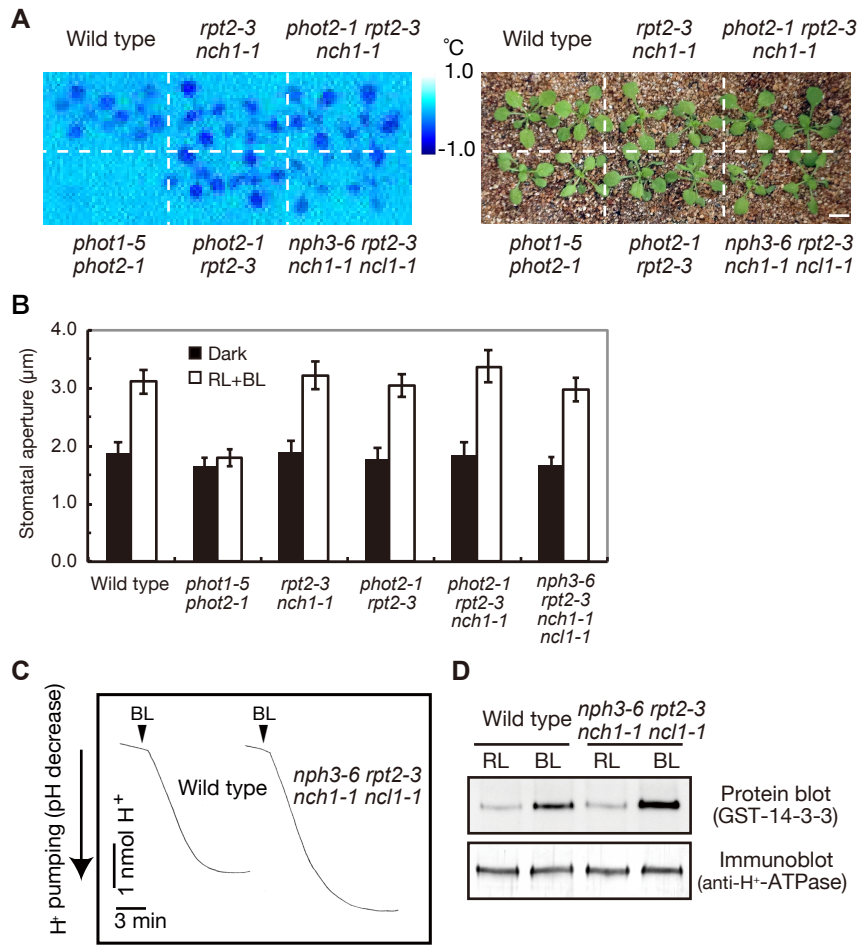


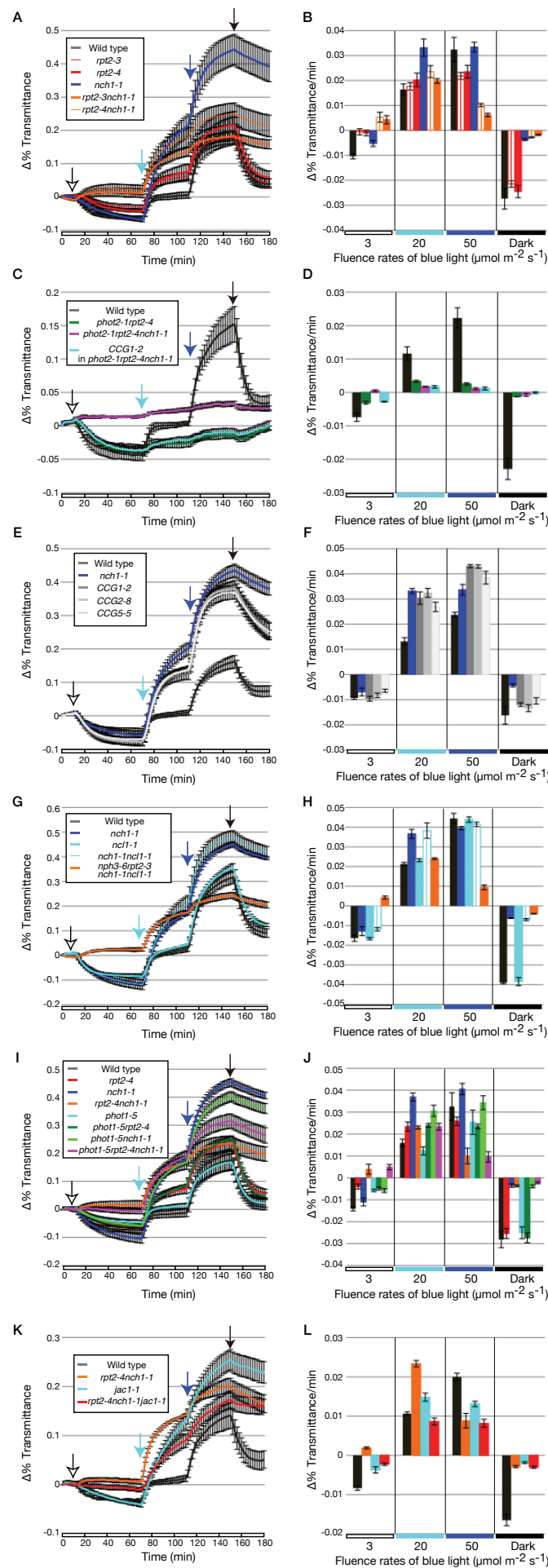
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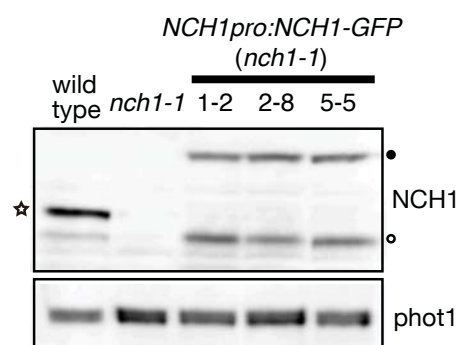
B











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MpNCH1    1  MAVSAKPEKIAVVRKPFRTSPTMKRTNHWVLATDVPDSVVVIVGCTSPALHKKPPLVSACGIRMLVVEAG-DSIVRQELPDIPGGAEGFIIAAKFCYGI
NCH1      1  -----MSAKKKDLISSAMKRTSFWISSQVSSDVVHVGGEASFLHKKPPLISKCGFIKKLVSESSKDSISITIKIPDIPGGSFPELAAKFCYGI
NCL1      1  -----EKKSLHKKPPLVSKCGFIKKLASESS--NISMIRIIPDPGGAEGFELVVKFCYGI

RPT2      95  NFEITIVCNVAADHCAAELEMTIKYCDNLAGRTQDPLSQVALSSLSGAVVVKSCBLLPLSRDLCVRRCDVVVGAKACN-----EAMF-----
MpNCH1    100  NFEITITANVAALRCAAEYLEMTEEYEGENLVARTEAALSEVVCNADSIIVLHNCENLLPABEIRKIVSRITDAIASKACREQIAQGMGBSDMDISGRN
NCH1      91  NFMSTENIAMLRCAAEYLEMTEEHSVENLVVRABALNVEALKSLSSSITVLHKSRLPLPAERVKLVSRCIDAIAYMTCC-----ESHHCSPSSS
NCL1      58  SFEINTENIAMLRCAAEYLEMTEEHSVENLVETIEVILNVEVLKSLSKMVKLCKSQDLLPIAERVRLVRCIDSIAYATCC-----ES-----

RPT2      181  -----PCHTTP-NMWTEELGILDVDFFSIVVSSMKQRCVVPSSLSAITYIEKSLRDLVRDHSGR----CKYSEPGDNESERSQORDLVQS
MpNCH1    200  DSNVKLSFSKGPQVTPSDWWAEDLAVLRIDFVORVLAAMRSRLAYBSICGALMHYAHSLGLHFKQGRDMYKCGOCKKQHDSTSAIEHEORILVET
NCH1      183  NSGNNEVVVQQSKCHVVDWAAEDLVLRIDSFORVLIAMARGFKQVGLGFLVLMLYAKSLRGL-----EIFGKGKKIKPKOEHEKRVILET
NCL1      142  -----CSNEDIVDWWAEDLAVLRIDMERRVLVAMTARGFKRYSGLGFLVLYAEKALRGLVFLNFLTEQCDFGKEAKKMBABOEHEKRLILET

RPT2      265  IVSLLFSTRGLPFPNFTCSLLRCVAFLETSITCKNLEKRISTVLEHVSVDLLIPSFTVDFE-RILDLSVRIISAVVEKEKNVGV-----S-----
MpNCH1    300  IVSLLFPEKNIASSPFLGQLRITATILTTIACRLDLERRGQLEQALDLDLLIPSSTIGDITFDVTVQRIIVCHTQQTINEDVHDPHSMBSGEG
NCH1      272  IVSLLPREKNASVSFSLMLLRAAIFLETTVACRLDLENRGLQLGAVLDDLLIPSSTIGDHSFDDDTVQRIIMNLE-----FEVEGV
NCL1      231  IVSLLPRERNSVSFSLMLLRAAIFLETTVACRLDLEKRGLOLRGAVDLDLLIPYSNGLDMIDVDTVQRIIMNLE-----FEVEG-

RPT2      353  ---NGEDNRGVCVSIVQRVAKIVDSYLAETIAYGLTITISKFNATIANVEKSARKSDDDIYRAIDIFLKAHPNLDETERKVCSSMDPIKLSYDARLHA
MpNCH1    398  ---MCSPTQS-----AMKVAKLLSYLAETIAPANLKLAKFISLAELFEYARVVDGLYRAIDIYLAHPSLETIDRKKICKLMDVQKLSQACATHA
NCH1      359  RLSNNGVCLAG-----DMERVGKLLSNYAEIASDRNVSLQKFIGLAELIFEOSRVTEGMYRAIDIYLAHPNMSDVERKKVCSLMDCCQKLSREACAH
NCL1      317  ---NSADFAS-----IGELMETYLAETIASDRNINFAKFIGABGIFKQSR-----MYRAIDIFLKAHPNISEVERKKVCSLMDCCQKLSRVYAHA

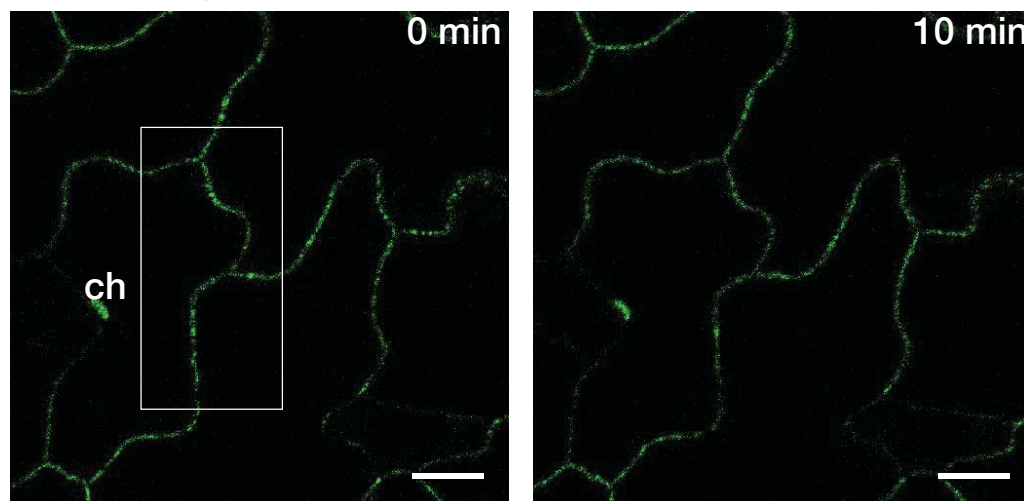
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MpNCH1    489  AQNDRLPVQIVQVLYFEQLRLRTATIGSLADVHVEHSCQITIGAFSSASP-RDNY---ASVRENRRLKLEVAEMMMLIDLEKHVCMKQDIPKGG
NCH1      454  AQNDRLPVQIVQVLYYEQRLRGENT---NDSDSPAFPPQOPAAVLPKLLSSYIDEI---SKLRENOQLKLELLKMKMKLKESEKSEKTSSTIST
NCL1      400  AQNDRFQENT-----SNSDSPAFAAAEKILSPPLSSYNEL---SKLRENOQLKLELLKMKMKLKESEKSEKTSSTIST

RPT2      529  AGASSSSSSIVSSKSHHFFSSVSKKLGKLINFFKNESRDTSHIDEDLGGVDITKF-----RRRRHSIS
MpNCH1    585  S-----GRFLLSVSKKLTLLNFFSKSEIKG-----GALDERAPNTPDSRQGGSGGRRRHSIS
NCH1      548  NPSSPLSTAGTKFELPRKSFINSVSKLGKLINFFSITPYN-----GRGRITKPF-----KRRRHSIS
NCL1      473  DCSSSVSTASVAKPRLPRKSFINSVSQKLGKLINFF---GKK---C---QTKPF-----KRRRHSIS

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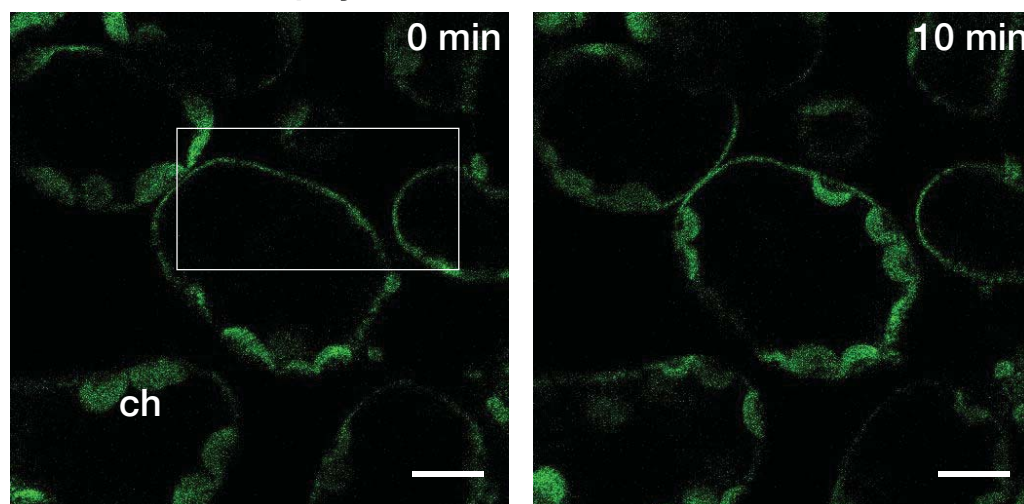
A

*A. thaliana* pavement cells



B

*A. thaliana* mesophyll cells



C

*M. polymorpha* thallus cells

